REMARKS

New Claims 24 and 25 have been added. Support for the subject matter of new Claims 24 and 25 may be found in the substitute specification, for example, at page 35, lines 3-10.

In the Office Action mailed March 21, 2004, the abstract has been objected to for not being on a separate sheet. The specification has been amended to include a separate sheet containing the abstract. Withdrawal of the objection to the specification is respectfully requested.

Claims 3-6 and 19-23 have been objected to as improperly dependent. Claims 1 and 17 have been amended to recite that the nucleotide incorporation is determined quantitatively, and Claims 3 and 19 have been cancelled without prejudice. Claims 4 and 20 have been amended to correct dependencies. Claim 9 is objected to as allegedly failing to further limit Claim 8. The Examiner has noted that Claim 1, upon which Claim 8 depends, has the limitation of a primer extension reaction. Applicant submits that Claim 9 refers to a primer extension reaction before pooling, and is distinct from the primer extension reaction recited in Claim 1. Claim 9 has been amended to clarify this distinction. Withdrawal of the objection to Claims 3-6, 9 and 19-23 is respectfully requested.

Claims 1, 3, 17 and 19 have been rejected under 35 U.S.C. § 102(b) as allegedly anticipated by U.S. Patent No. 6,020,137 to Lapidus et al. ("Lapidus et al."). The Examiner has alleged that Lapidus et al. teach a method for detecting loss of heterozygosity in a pooled patient population comprising: pooling samples; hybridizing probes to the sample which are immediately adjacent to a single base polymorphism; exposing the pooled samples to a plurality of different dideoxynucleotides; washing the sample; determining which of the dideoxynucleotides are incorporated into the probes; and detecting the incorporated dideoxynucleotide at the site.

Applicant respectfully submits that Lapidus et al. fail to teach every element of the claimed invention, and therefore fail to anticipate the subject matter of Claims 1, 3, 17 and 19. The methods of Claims 1 and 17 require the step of "sequentially adding nucleotides to the

reaction mixture and determining the incorporation or non-incorporation of each nucleotide as each nucleotide is added." Lapidus et al. do not teach or suggest this step of sequential nucleotide addition and real-time detection of incorporation. Rather, Lapidus et al. disclose a method in which at least two dideoxynucleotides are added simultaneously. Lapidus et al. at Col. 5, lines 49-56. Accordingly, Lapidus et al. fail to anticipate the present invention.

In addition, the nucleotides that are sequentially added in the method of the present invention are non-chain terminating nucleotides. The method of Lapidus et al. utilizes chain terminating dideoxynucleotides. Lapidus et al. fail to disclose a method in which non-chain terminating nucleotides are sequentially added, and incorporation or non-incorporation is determined as each nucleotide is added. Accordingly, withdrawal of the rejection of Claims 1, 3, 17 and 19 under 35 U.S.C. § 102(b) is respectfully requested.

Claims 4-7 and 20-23 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by WO98/28440 to Nyren et al. ("Nyren-2") in view of Lapidus et al. The Examiner has alleged that Nyren-2 teaches a method of sequencing DNA based on the detection of release of pyrophosphate. Further, the Examiner has alleged, Nyren-2 teaches a method of identifying a base at a target position using an extension primer that hybridizes immediately adjacent to the target position, wherein deoxynucleotides are added, and incorporation is detected by the release of pyrophosphate. It would have been obvious, the Examiner has alleged, to modify the method of Nyren-2 by pooling the nucleic acids as taught by Lapidus et al.

Applicant respectfully submits that there would have been no motivation to combine Nyren-2 with Lapidus et al. Nyren-2 teaches only a method of sequencing DNA, and thus one would have been motivated to use a homogenous nucleic acid sample, or a heterozygous sample from a single individual, in such a method, not a pooled sample. Nyren-2 does not teach or suggest that the method disclosed therein would be useful for any purpose other than sequencing and sequencing-related applications such as the detection of single base changes.

As motivation to combine the references, the Examiner points to the uses of pooled samples disclosed by Lapidus et al. at Col. 4, lines 35-40. However, the mere fact that pooled samples have certain uses does not provide motivation to use pooled samples in the method of Nyren-2. To the contrary, one would have expected that the method of Nyren-2 would be

suitable only for a homozygous sample or a heterozygous sample from a single individual. See Nyren-2 at page 18, lines 13-27.

In addition, it was known at the time of filing of the present application that the detection of genetic changes such as SNPs or loss of heterozygosity in pooled samples requires DNA methods capable of highly accurate and quantitative analysis. For example, Lapidus et al. disclose that "it is important to count the number of molecules in order to provide a statistical analysis of the likelihood of loss of heterozygosity." Col. 12, lines 28-34. Lapidus et al. also teach that statistically significant differences between relative proportions of the respective nucleic acids being compared need to be determined (Col. 6, lines 23-31; Col. 8, lines 2-4.)

Thus allele frequency determination requires a method that has high detection sensitivity and specificity. Many methods of sequencing or base detection are not sufficiently reliable or consistent, or do not provide sufficiently quantitative discrimination on a pooled sample. For example, methods such as traditional Sanger sequencing are not sufficiently accurate and quantitative for such a determination. Nyren-2 simply provides another method of sequencing DNA, and there is no indication that such a method would be quantitative enough for use in allele frequency determination using pooled samples. Accordingly, one would not have been motivated to use the sequencing method of Nyren-2 in a pooling application that requires a much more detailed and accurate quantification. Nor would one have had a reasonable expectation of success in achieving the requisite level of quantitation using the method of Nyren-2 with pooled samples.

It is only in accordance with the present invention that it was surprisingly discovered that a bioluminetric pyrophosphate method was quantitative enough to accurately detect allele frequencies in pools of samples. As disclosed in the substitute specification at page 37, lines 8-15, the correlation between peak heights and allele frequencies has been shown to be "excellent," and the accuracy of the results obtained "surprisingly high." It is impermissible now, with the benefit of hindsight and applicant's invention, to pick and choose elements of the cited references when one of ordinary skill in the art would not have been motivated to combine the elements.

Even if one of ordinary skill in the art had been motivated to combine Nyren-2 and Lapidus et al., the combination thereof fails to achieve the present invention. The references, alone or in combination, fail to teach a step of determining the frequency of an allele from a pattern of nucleotide incorporation. Lapidus et al. do not determine frequency of an allele, but rather measure dideoxynucleotide incorporation to determine whether normal heterozygosity has been lost by deletion. Lapidus et al. at Col. 5, lines 61-67. Accordingly, the combination of Nyren-2 and Lapidus et al. fails to render the present invention obvious.

In view of the foregoing comments and amendments withdrawal of the rejection of Claims 4-7 and 20-23 under 35 U.S.C. § 103(a) is respectfully requested.

Claims 8-11, 14 and 15 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by Lapidus et al. in view of Breen et al. (March 2000) BioTechniques 28:464 ("Breen et al."). As discussed hereinabove, Lapidus et al. fail to teach or suggest the step of sequential addition of non-chain terminating nucleotides. Breen et al. fail to remedy this deficiency, and thus the combination of references fails to render the present invention obvious.

Submitted herewith is the Declaration under 37 C.F.R. § 1.132 of inventor Anna Sylvan evidencing a date of invention prior to the publication date of Breen et al. Accordingly, withdrawal of the rejection under 35 U.S.C. § 103(a) over Lapidus et al. in view of Breen et al. is respectfully requested.

Claims 8-15 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by Lapidus et al. in view of Germer et al. (2000) Genome Research 10:258 ("Germer et al."). The Examiner has alleged that it would have been obvious to modify the method of Lapidus et al. with the teachings of Germer et al. that concentrations of samples should be ensured and that using the same concentration of each DNA sample is important. Germer et al. do not teach adjusting the amount of nucleic acids to substantially the same amount, but the Examiner has alleged that such an adjustment would have been obvious.

As discussed hereinabove, Lapidus et al. fails to teach or suggest the step of sequentially adding non-chain terminating nucleotides. Germer et al. fail to remedy this deficiency, and thus the combination of Lapidus et al. and Germer et al. fails to render obvious the present invention. Further, Germer et al. simply does not teach or suggest a step of adjusting the concentration of

nucleic acids in each sample of the population which is pooled. The test proposed by Germer et al. only shows whether the method as a whole gives accurate enough measurement on the pool. It does not show how an individual sample deviates, or which samples will require adjustment prior to pooling. Further, Table I of Germer et al. shows the general accuracy of the method, and that the data is linear over all tested frequencies. It does not, however, serve to construct a calibration curve, and cannot be used for DNA calibration, as it does not provide information on individual samples.

Accordingly, the present invention is not rendered obvious by Lapidus et al. in view of Germer et al., and withdrawal of the rejection under 35 U.S.C. § 103(a) is respectfully requested.

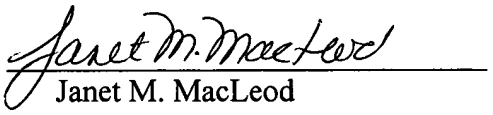
In view of the foregoing comments and amendments, it is respectfully submitted that the present application is in condition for allowance.

Entry of the present amendment, and favorable consideration and allowance of all pending claims is earnestly solicited.

Respectfully submitted,

DORSEY & WHITNEY LLP

Date: August 26, 2004



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4841-9631-10401

DOCKET 14255.01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

First Named Inventor:	Anna Sylvan	
Application No.:	10/085,774	
Filing Date:	February 27, 2002	Examiner:
Title:	METHOD FOR DETERMINING ALLELE FREQUENCIES	Group Art Unit:

DECLARATION UNDER 37 CFR § 1.131

I, ANNA SYLVAN, hereby declare as follows:

1. I am the sole inventor of the invention disclosed and claimed in U.S. Patent Application Serial No. 10/085,774 filed February 27, 2002, which claims priority under 35 U.S.C. § 119(e) from U.S. Patent Application Serial No. 60/271,703 filed February 27, 2001.

2. At the time the invention was made, I was employed by Pyrosequencing AB, the assignee of the above-identified application. I continue to be employed by Pyrosequencing AB.

3. I conceived and reduced to practice the subject matter of the invention claimed in the 10/085,774 application prior to March 2000. In particular, I conceived and reduced to practice the method of determining the frequency of an allele in a population of nucleic acid molecules by pooling the nucleic acid molecules, performing primer extension reactions using a primer that binds to a predetermined site in the molecules, and obtaining a pattern of nucleotide incorporation.

4. As evidence of the conception and reduction to practice of the invention prior to March 2000, attached hereto as Exhibit A are copies of seven pages of my laboratory notebook. Each of the dates redacted from the pages of the laboratory notebook is prior to March 1, 2000.

5. The laboratory notebook pages demonstrate that I conceived and reduced to practice the method of the invention to calculate allele frequencies by pooling nucleic acid molecules, performing primer extension reactions using a primer that binds to a predetermined site in the molecules, and obtaining a pattern of nucleotide incorporation. For example, at page 32 of my notebook is the protocol for an experiment to determine the allele frequencies for single nucleotide polymorphisms (SNPs) designated Eu1, Eu4 and Eu7 using primers designated E1s, E4s and E7s. These SNPs and primers are described in the 10/085,774 application at pages 56-57. Page 33 of the laboratory notebook shows the results in the form of nucleotide incorporation versus amount of light released in the Pyrosequencing reaction. Page 34 of the laboratory notebook shows that when peak heights were plotted against allele frequency, a linear relationship was demonstrated.

6. Pages 35-38 of my laboratory notebook attached hereto as Exhibit A demonstrate an experiment in which polymerase chain reaction (PCR) products from 47 individual samples were genotyped for two different SNPs designated Aop 52 and Aop 54 (pages 35 and 36), whereafter they were pooled (page 37), and allele frequency was calculated based on the pattern of nucleotide incorporation (page 38).

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements are made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing thereon.

13 Jan, 2004
Date

Anna Sylvan

Notebook Number: Anna Sylvan ①

Date: REDACTED

Känslighetsförsök 1

	Läst	Forward			
Eu1 = ACP-240	(A/T)	A/T ✱✱	158 bp	Eu1:1 = A	Eu1:2 = T
Eu4 = ACEex15	(A/G)	A/G	145 bp	Eu4:1 = A	Eu4:2 = G
Eu7 = ANP1218	(C/T)	G/A	142 bp	Eu7:1 = T	Eu7:2 = C

		spädning (0.1 pmol/µl)			
Eu1:1	0.17 pmol/µl	353	+	247	H_2O
Eu1:2	0.14	429	+	171	
Eu4:1	0.26	231	+	369	
Eu4:2	0.18	333	+	267	
Eu7:1	0.16	375	+	225	
Eu7:2	0.16	375	+	225	

Följande spädningsserie gjordes:

Volymer i µl.

ANP (0.1 pmol/µl)	100% prov 1	98% prov 1	95% prov 1	90% prov 1	85% prov 1	80% prov 1	75% prov 1	70% prov 1	65% prov 1	60% prov 1	55% prov 1	50% prov 1	45% prov 1	40% prov 1	35% prov 1	30% prov 1
Eu1:1	50	49	47,5	45	42,5	40	37,5	35	32,5	30	27,5	25	22,5	20	17,5	15
Eu1:2	0	1	2,5	5	7,5	10	12,5	15	17,5	20	22,5	25	27,5	30	32,5	35
Eu4:1	50	49	47,5	45	42,5	40	37,5	35	32,5	30	27,5	25	22,5	20	17,5	15
Eu4:2	0	1	2,5	5	7,5	10	12,5	15	17,5	20	22,5	25	27,5	30	32,5	35
Eu7:1	50	49	47,5	45	42,5	40	37,5	35	32,5	30	27,5	25	22,5	20	17,5	15
Eu7:2	0	1	2,5	5	7,5	10	12,5	15	17,5	20	22,5	25	27,5	30	32,5	35

25% prov 1	20% prov 1	15% prov 1	10% prov 1	5% prov 1	2% prov 1	0% prov 1
12,5	10	7,5	5	2,5	1	0
37,5	40	42,5	45	47,5	49	50
12,5	10	7,5	5	2,5	1	0
37,5	40	42,5	45	47,5	49	50
12,5	10	7,5	5	2,5	1	0
37,5	40	42,5	45	47,5	49	50

Till varje brunn/provblandning tillsattes 17.5 µl (10 µg/µl) magnetkulor. Inbindning 15 min, 65°C, 1400 RPM

Tvättning i 0.5 M NaOH (ca. 1 min)

Tvätt i 1x annealing buffert.

5 pmol selverensprimer (E1S, E4S resp. E7S) i 40 µl annealingbuffert. Annealing 80°C, 2 min

Sekvenskit: 01 0003 Kassett: Marknad 1 (andra körningen)

Dokument: Serie 1

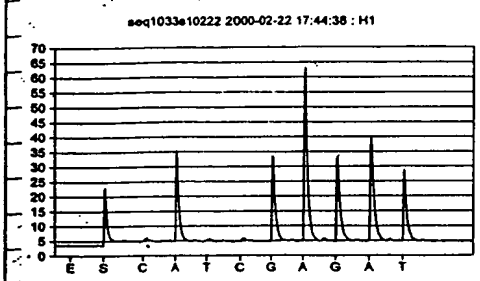
Filnamn: Sg1033$10222

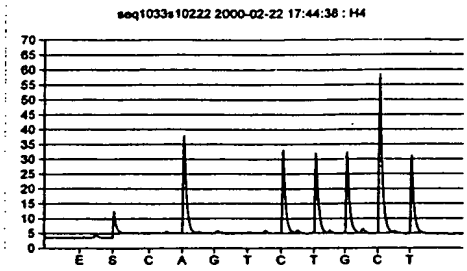
Forts →

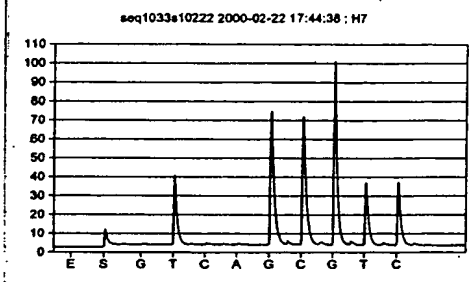
Read and Understood By

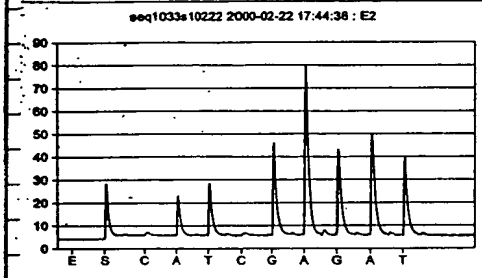
Forts. från sid 32

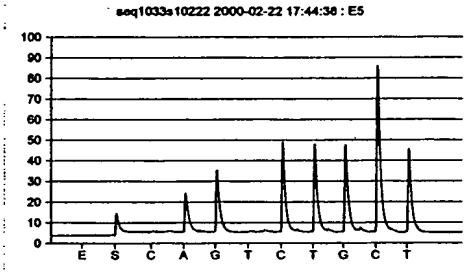
Resultat: Det ser mycket lovande ut! Dock var inte de 2 homozygot proverna jämnstarka. Försöket bör göras om med korrigerade spädningar.

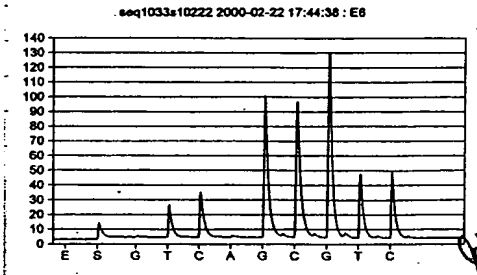


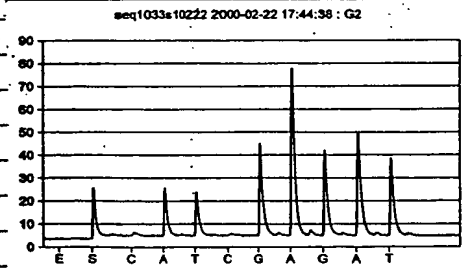


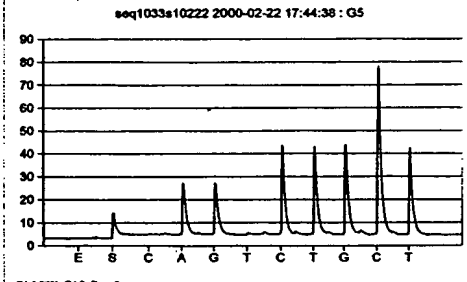


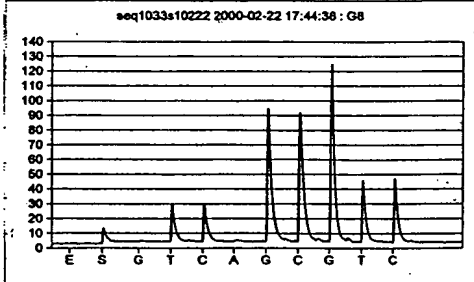


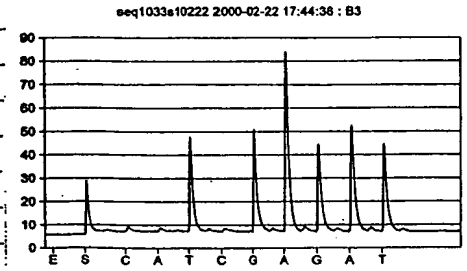


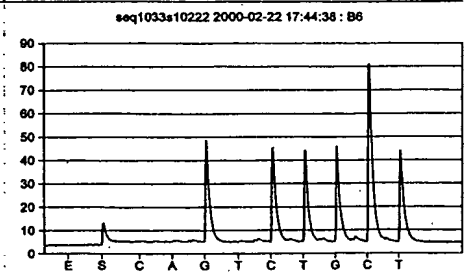


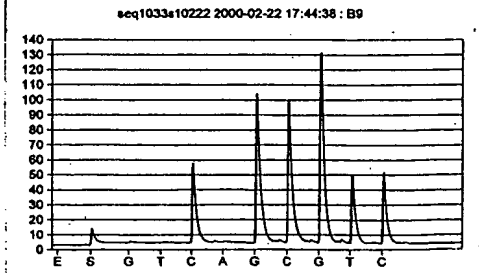












Ursprungs-koncentrationerna för E1:2, E4:2 och E7:2 kompenseras enl. följande: E1:2 $\frac{600 \cdot 0.138}{429} = 0,193$

E4:2 $\frac{600 \cdot 0.15}{333} = 0,27$ E7:2 $\frac{600 \cdot 0.144}{375} = 0,23$

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Notebook Number: Anna Sylvan ①

Date: REDACTED

Känslighetsförsök 2

Upprepning av försöket på sid 32-33 med korrigerade PCR-koncentrationer för Eu1:2, Eu4:2 och Eu7:2.

		spädning (till 0.1 pmol/µl)	
Eu1:1	0.17 pmol/µl	353 µl prov +	247 µl H_2O
Eu1:2	0,19	311	289
Eu4:1	0.26	231	369
Eu4:2	0.27	222	378
Eu7:1	0.16	375	225
Eu7:2	0.23	261	339

Samma spädningsserie gjordes som på sid 32.

17,5 µl magnetkulor (10µg/µl) per reaktion tillsattes direkt (utan tvätt!) Inbindning 15 min, 65°C, 1400 RPM

Denaturering ca. 1min, 0.5 M NaOH

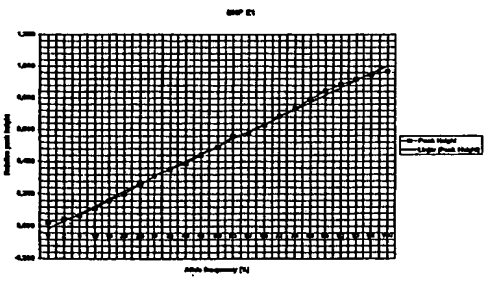
1 tvätt i 1× Anneal buff. Annealing i 40 µl 1× AB med 15 pmol primer, 80°C, 2 min. (Selv. primer E1s, E4s o E7s)

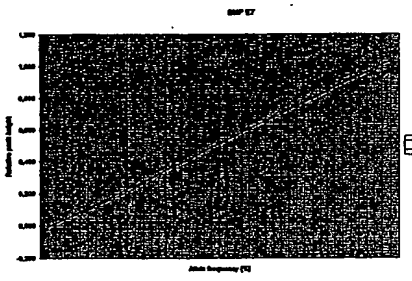
Sekvenslist: 0.0003 Filnamn: seq_1034s_0223

Instrument: Serie 1

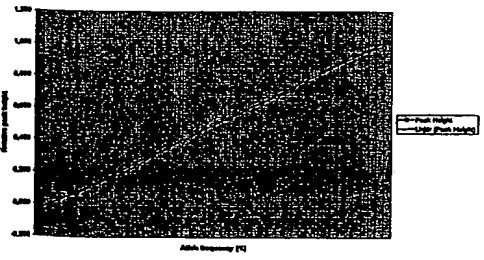
Kassett: Marknad 1 (3:e körningen) SNP method ver. 1.0

Resultat: Lysande! Relativa topphöjder (homo 1)/(homo 1+homo 2) mot allelfrekvens ger linjärt samband!





← OBS! Körningen kunnorms endast bort på att Excel sparar data felaktigt (...95,98,100)



← Disp. fel för punkten 50:50% ger ett något avvikande mätvärde.

Read and Understood By

AstraZeneca SNP 52 o 54

Syfte: Att köra kundprover från AstraZeneca (Hässle) på PCR produkter som erhållits från kunden. SNP:s Aop 52 och Aop 54 för 47 prover + PCR nollor.

Tyroseq 00-02-22

	1	2	3	4	5	6	7	8	9	10	11	12
A	522	156	398	668	617	842	522	156	398	668	617	842
B	860	521	49	742	679	851	860	521	49	742	679	851
C	745	321	246	1139	813	639	745	321	246	1139	813	639
D	467	1168	450	847	932	1140	467	1168	450	847	932	1140
E	592	426	390	706	841	184	592	426	390	706	841	184
F	599	1041	397	440	855	940	599	1041	397	440	855	940
G	449	104	271	659	892	889	449	104	271	659	892	889
H	346	245	463	375	908	neg kont.	346	245	463	375	908	neg kont.
		Aop52						Aop54				

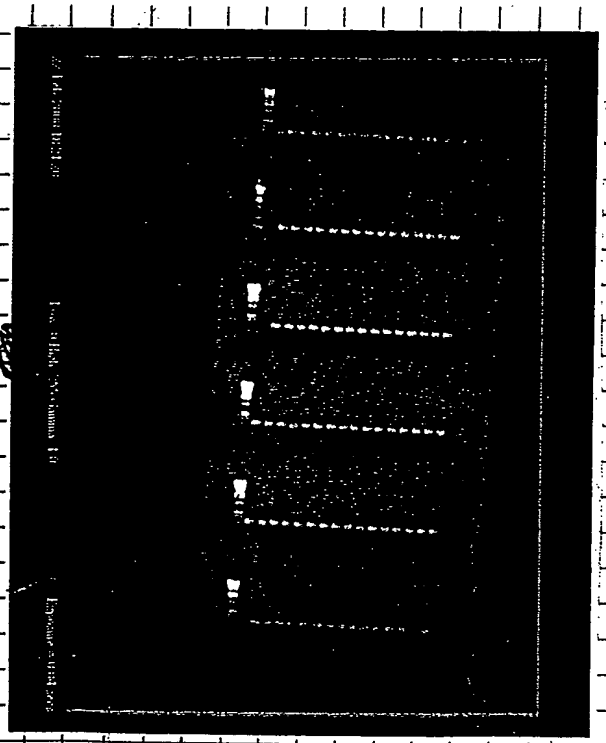
PCR-mix		x 50
10x buffert	10	500
25 mM Mg	12	600
2.5 mM dNTP	8	400
primer 10 pmol/ul	2	100
primer 10 pmol/ul	2	100
Amp Taq Gold	0.4	20
Templat 10 ng/ul	2	100
vatten	63.6	3180
	100	5000

primer par
M-4-BIO+Aop51

Aop25-BIO+Aop53

PCR program

95 C	5 min	
95 C	30 sec	
64 C	45 sec	x 50
72 C	1 min	
72 C	5 min	
8 C	forever	



25 µl PCR produkt + 9 µl magnet-kulor (10 µg/µl) (otvättade kulor direkt från burken) + 16 µl 2×BW (⇒ totalvolym 50 µl). Inbindning 15 min, 65°C, 1400 RPM.
Denaturering ca. 1 min i 0.5 M NaOH. 1 tvätt i 1×AB.
Annealing 80°C, 2 min, i 45 µl AB med 15 pmol primer.
Reagenslot: 010003
Instrument: Serie 1
SNP method ver. 1.0
Kassett: Marknad 2 (Första körningen)

Filnamn: seq1035s10224

Forts →

Signed

Read and Understood By

Signed

Notebook Number: Anna Sylvan (1)

Date: REDACTED

Forts från sid 35

Resultat: Fina kurvor o signaler! Samtliga prover genotypade med hög kvalitet!

Genotyper erhållna med TaqMan

Pyrosekvensprover

TVP	pol 1	pol 2
522	2	2
980	2	2
745	2	2
467	2	2
883	1 and 2	1 and 2
599	1 and 2	1 and 2
449	1 and 2	1 and 2
849	2	2
156	1 and 2	1 and 2
821	1 and 2	1 and 2
321	1 and 2	1 and 2
1168	2	2
426	2	2
1041	2	2
104	2	2
943	1 and 2	1 and 2
396	2	2
49	1 and 2	1 and 2
846	1 and 2	1 and 2
460	1 and 2	1 and 2
390	1 and 2	1 and 2
397	2	2
271	2	2
483	2	2
899	1 and 2	1 and 2
742	2	2
1139	2	2
847	2	2
708	2	2
440	1 and 2	1 and 2
959	2	2
375	2	2
917	1 and 2	1 and 2
979	2	2
813	1 and 2	1 and 2
932	1 and 2	1 and 2
841	1 and 2	1 and 2
855	2	2
992	2	2
908	1 and 2	1 and 2
942	1 and 2	1 and 2
851	2	2
639	1	1
1140	1	1
184	2	1 and 2
940	2	2
999	1	1

pol 1 allel 1= A allel 2= G

pol 2 allel 1= C allel 2= T

Genotyper m. Pyro (seq1085 s10224)

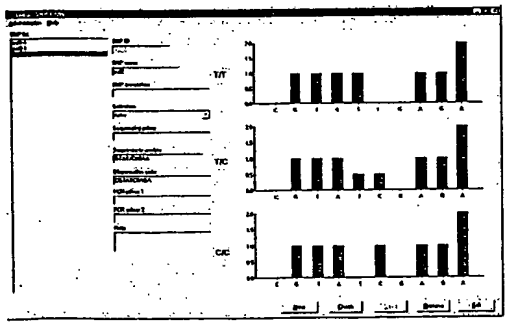
Resultat för Aop52

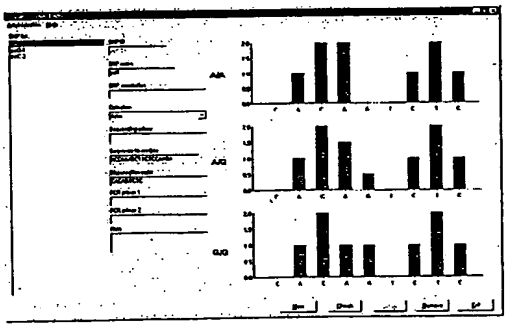
A1:	522	G/G
A2:	156	A/G
A3:	396	G/G
A4:	668	A/G
A5:	817	A/G
A6:	842	A/G
B1:	860	G/G
B2:	821	A/G
B3:	49	A/G
B4:	742	G/G
B5:	879	G/G
B6:	851	G/G
C1:	745	G/G
C2:	321	A/G
C3:	240	A/G
C4:	1139	G/G
C5:	813	A/G
C6:	639	A/A
D1:	467	G/G
D2:	1168	G/G
D3:	450	A/G
D4:	847	G/G
D5:	932	A/G
D6:	1140	A/A
E1:	582	A/G
E2:	426	G/G
E3:	380	A/G
E4:	708	G/G
E5:	841	A/G
E6:	184	G/G
F1:	569	A/G
F2:	1041	G/G
F3:	397	G/G
F4:	440	A/G
F5:	855	G/G
F6:	940	G/G
G1:	449	A/G
G2:	104	G/G
G3:	271	G/G
G4:	858	G/G
G5:	892	G/G
G6:	889	A/A
H1:	346	G/G
H2:	245	A/G
H3:	463	G/G
H4:	375	G/G
H5:	908	A/G
H6:	nolla	

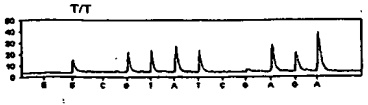
Resultat för Aop54

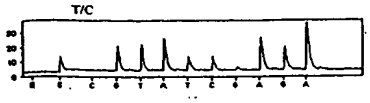
A7:	522	T/T
A8:	156	T/C
A9:	396	T/T
A10:	668	T/C
A11:	817	T/C
A12:	842	T/C
B7:	860	T/T
B8:	821	T/C
B9:	49	T/C
B10:	742	T/T
B11:	879	T/T
B12:	851	T/T
C7:	745	T/T
C8:	321	T/C
C9:	240	T/C
C10:	1139	T/T
C11:	813	T/C
C12:	639	C/C
D7:	467	T/T
D8:	1168	T/T
D9:	450	T/C
D10:	847	T/T
D11:	932	T/C
D12:	1140	C/C
E7:	582	T/C
E8:	426	T/T
E9:	380	T/C
E10:	708	T/T
E11:	841	T/C
E12:	184	T/C
F7:	569	T/C
F8:	1041	T/T
F9:	397	T/T
F10:	440	T/C
F11:	855	T/T
F12:	940	T/T
G7:	449	T/C
G8:	104	T/T
G9:	271	T/T
G10:	858	T/T
G11:	892	T/T
G12:	889	C/C
H7:	346	T/T
H8:	245	T/C
H9:	463	T/T
H10:	375	T/T
H11:	908	T/C
H12:	nolla	T/T

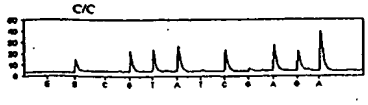
Resultaten överensstämmer väl! (100%)

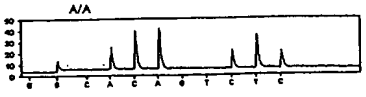


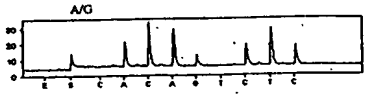


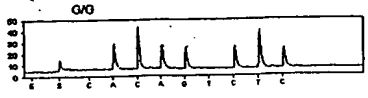












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Allelfrekvens i poolad population

Syfte: Att poola 47 olika prover och i en enda pyro-körning beräkna populationens allelfrekvens för den testade SNP:n

Använde proverna/SNP:arna från sid 35 (Aop52 och Aop54)

Allelfrekvenser: Aop 52 73,4% G, 26,6% A

Aop 54 72,3% T 27,7% C

Aop52: 5µl av vardera prov blandades ihop (⇒ tot. vol. 235µl)

Tillsats av 5×17,5µl (10µg/µl) kulor + 5×32,5µl 2×BW.

Blandning genom vortex. Utspädning av 100µl i 4 rör o 85µl i det sista.

Aop54: 6µl av vardera PCR prod. blandas (⇒ 282µl). 250µl av detta blandades med 5×17,5µl kulor + 5×32.5 µl 2×BW-buffert. Vortex.

Utspädning av 100 µl i 5 rör.

Inbindning Aop52: 15min, 65°C, 900 RPM + 15min, 65°C, 1400 RPM

Aop54: 15min, 65°C, 1400 RPM

Denaturering ca. 1 min i 0,5 M NaOH

Tvätt i 1×AB. ~~Hyb~~ Annealing till 15 pmol primer i 45µl annealingbuffert. 80°C i 2 min.

Sekvenskit: 010003 SNP Method Ver 1.0

Instrument: Serie1

Kassett: T2 Filnamn: seq1036s10225

fortsättning ⟶

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Notebook Number: Anna Sylvan (1)

Date: REDACTED

forts. från sid 37

Resultat: Mycket bra! Dock, A-topparna betydligt högre än övriga toppar (~20%!). Varför? Pga kitet 010003 (som vi vet har stabilitetsproblem)?

Anna Sylvan

Från: Anna Sylvan [anna.sylvan@pyrosequencing.com]
Skickat: den 26 februari 2000 18:07
Till: 'Ingemarsson, Björn'; 'Ekström, Björn'; 'Nilshans, Helena'; 'Winge, Mårten'
Ämne: Allelfrekvensbestämning

Hej,

Här kommer lite nya trevliga resultat. Jag har gjort ett första försök med allelfrekvensbestämning i en poolad provpopulation (47 individer) för 2 olika SNPar.

För vardera SNP gjordes följande: Till poolningen användes 6 ul PCRprodukt från vardera prov. Satte sedan 5 st replikat á 50* ul av poolen (för att få höga signaler). Provprep med förenklat protokoll.

*I replikat 1 för SNP 2 har endast 35ul av poolen använts vilket leder till ngt lägre total signalnivå i denna.

SNP 1: Enkel T/C polymorfi med jämn topphöjd för heterozygoter (50:50). Allelfrekvens i den använda populationen: **72.3% T, 27.7%C**

Allelfrekvensen i poolade proven beräknades enligt: $\%C = topphöjd_C/(topphöjd_C + topphöjd_T)*100$

Resultat: Lysande överensstämmelse med förväntad allelfrekvens!

Replikat	SNP1	Topphöjd	%C
Rep1	T	25,73	27,72
	C	9,87	
Rep2	T	27,37	27,67
	C	10,47	
Rep3	T	25,88	28,11
	C	10,12	
Rep4	T	25,28	27,81
	C	9,74	
Rep5	T	22,38	27,31
	C	8,41	
		Average	**27,7**

SNP 2: Betydligt svårare test. A/G polymorfi som kommer efter ett A i sekvensen => A/A 2A:0G, A/G 1.5A:0.5G, G/G 1A:1G. Heterozygoten är dessutom något skev (ingen A-toppskorrigering görs i PyroAnalyzer!) med synbar allelfrekvens 43%G, 57% A istället för 50:50% i heterozygota prover. Allelfrekvens i den använda provpoolen: **73.4% G, 26.6% A**

Allelfrekvensen i poolade proven beräknades först rakt av enligt: $\%G = 2*topphöjd_G/(topphöjd_G + topphöjd_A)*100$

$\%A = (topphöjd_A - topphöjd_G)/(topphöjd_G + topphöjd_G)*100$

Korr 1: Om man antar att heterozygoten är skev enbart pga för höga A-toppar kan följande korrigering göras (0.83 har jag räknat fram utifrån topphöjden hos äkta heterozygoter för att tvinga %G till 50% för dessa):

$\%G\ korr.1 = 2*topphöjd_G/(topphöjd_G + 0.83*topphöjd_A)*100$

Korr. 2: Om man istället antar att A-topparna generellt har samma höjd som övriga toppar och att skevheten snarare beror på verklig skevhet i PCR-amplifieringen av de två allelerna (vilket jag tror är ett ganska ovanligt fenomen) så vore följande korrigering mer lämplig:

$\%G\ korr.1 = 2*topphöjd_G/(topphöjd_G + topphöjd_A)*100*(0.5/0.43)$

Resultat: Korrigering enligt alternativ 1, dvs. generell reducering av A-toppar utifrån heterozygoternas värden, ger lysande överensstämmelse med den förväntade allelfrekvensen i populationen!

	SNP 2	Topphöjd	%G	%G korr.1	%G korr.2
Rep 1	A	29,24	65,19	73,63	75,47
	G	14,14			
Rep 2	A	41,96	66,20	74,69	76,63
	G	20,76			
Rep 3	A	42,11	64,60	73,00	74,78
	G	20,09			
Rep 4	A	44,29	64,62	73,02	74,80
	G	21,14			
Rep 5	A	41,11	64,59	72,99	74,77
	G	19,61			
		Average	65,0	73,5	75,3

0.92 → 1.02

0.43 → 0.50

Hoppas att ni kan följa mina beräkningar –annars är det bara att höra av sig.

Hälsningar Anna

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